

Assay for Homocysteine

The invention relates to an assay for homocysteine in body fluids or fluids derived therefrom and to kits for such assays.

Homocysteine is a sulphur-containing amino acid that is closely related to methionine and cysteine. There is no DNA-coding for homocysteine and it is not present in naturally occurring proteins. Homocysteine is formed in the metabolism of the essential amino acid methionine. In plasma about 1% of homocysteine exists in the free reduced form, about 70% is bound to albumin and the rest is present in low molecular weight disulphides, predominantly with cysteine. Total homocysteine, a term describing all molecular forms of homocysteine (e.g. free and bound) is abbreviated hereinafter as tHcy. In the literature the term homocysteine is often abbreviated as Hcy and used to describe both free and bound homocysteine.

Hyperhomocysteinemia is generally defined as fasting plasma tHcy above 15  $\mu\text{mol/L}$ . It has been shown to be a risk factor for cardiovascular disease and for complications in pregnancy and congenital malformations. A link between impaired homocysteine metabolism and neuropsychiatric disorders and cognitive impairment in the elderly has also been shown.

Normalisation of homocysteine levels may be achieved by life-style changes (e.g. quitting smoking, taking exercise, reducing coffee consumption, improving diet, etc.) or by vitamin supplementation.

Accordingly there has been significant emphasis in recent years on the development of assays for homocysteine. Such assays are available for example from Abbott Laboratories (US), Axis-Shield (UK and NO), Bio-Rad (NO), and A/C Diagnostics (US). Such assays are moreover described in patent applications from Axis-

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Shield (NO) and the University of Glasgow (UK), e.g. US-A-5631127 and WO98/07872.

Direct determination of homocysteine is not straightforward and these assays generally involve enzymatic conversion of homocysteine and direct or indirect determination of a homocysteine conversion product. Thus for example US-A-5631127 (European equivalent EP 0 726 322) describes conversion of homocysteine and adenosine to S-adenosyl homocysteine (SAH) using SAH-hydrolase and determination of SAH, while WO98/07272 describes the conversion of homocysteine and water to  $\alpha$ -ketobutyrate and hydrogen sulphide using homocysteine desulphurase and determination of either the  $\alpha$ -ketobutyrate or the hydrogen sulphide.

JP 4329357 of Sinotest KK describes a general immunoassay method, using various compounds to prevent non-specific antibody interactions. Further, EP-A-483512 of Boehringer Mannheim GMBH describes immunological precipitation assays for analytes, whilst US-A-4298592 of Lin et al describes a double antibody separation methods.

For homocysteine to be available as an analytical parameter for most clinical laboratories however, a homocysteine assay is needed which may be performed in the commercially available automated clinical chemistry analysers, sometimes referred to as clinical chemistry platforms, for example the systems available from Hitachi and Roche Diagnostics.

Generally clinical chemistry analysers are characterised by the use of simple photometric measurements, e.g. colorimetric, turbidimetric or nephelometric measurements. Additionally the number of manual handling steps for running assays on these analysers have to be as few as possible. Preferably little or no sample pre-treatment should be required and ideally the reagents used should be assay-ready in a

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stable form. The number of compartments for reagents in these analysers is typically extremely limited, and since the analysers may be arranged to determine several different analytes in a sample, often only two or three reagent compartments may be available for an assay for a single analyte.

Furthermore, the total assay time required per sample must be relatively short both to optimise

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homocysteine in said sample; and photometrically detecting said complex.

The assay of the invention may be used to determine tHcy or free homocysteine; in the former and more usual case the sample will generally be treated with a reducing agent to liberate bound homocysteine.

By "stable" it is meant that the aqueous reagents should be capable after storage at 20°C for at least 3 months, preferably at least 6 months, more preferably at least 18 months, of being used to determine homocysteine content in a 10 µmol/L L-homocysteine standard with a loss in accuracy of no more than 10% preferably no more than 5% as compared with freshly prepared equivalent reagents. In testing stability, determination of the antibody:polyhapten complex may be performed turbidimetrically using a Hitachi 911 apparatus.

Viewed from a further aspect the invention also provides a homocysteine assay reagent kit comprising two or three stable aqueous reagents containing a polyhapten, a homocysteine converting enzyme, a primary antibody capable of binding to said polyhapten whereby to produce a complex, and if desired one or more of a co-substrate for said homocysteine converting enzyme, a reducing agent, a further enzyme capable of converting said co-substrate or a conversion product of said homocysteine converting enzyme, and a secondary antibody capable of binding to said complex, said primary antibody also being capable of binding to said co-substrate or a conversion product of one of said enzymes.

In the method of the invention, the homocysteine content of the sample, preferably the tHcy value, is determined indirectly by determining the amount of the polyhapten:antibody complex, preferably by nephelometry or turbidimetry. The homocysteine content may be determined quantitatively, e.g. in absolute units such as µmol/L, or alternatively the determination may be

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qualitative, e.g. simply that it is above a predetermined threshold such as 15, 18 or 20  $\mu\text{mol/L}$ . Generally, the assay measurement will be calibrated against standard homocysteine solutions containing known concentrations of homocysteine, usually L-homocysteine; however for assays run on automated analysers only occasional calibration will be necessary, e.g. when reagent reservoirs are refilled.

Where an antibody is used in the assay method and kits of the invention, this may be a polyclonal or more preferably a monoclonal antibody; however the term antibody is also used to cover single chain antibodies, antibody fragments (e.g. Fab fragments), and oligopeptides and oligonucleotides capable of highly specific binding to the polyhapten or polyhapten:primary antibody complex. Appropriate such specific binding oligonucleotide and oligopeptides may be identified by conventional combinatorial chemistry techniques, e.g. using phage display libraries. Antibodies to the hapten in the polyhapten, ie. to the direct or indirect homocysteine conversion product of the homocysteine conversion enzyme, the co-substrate, or to the polyhapten: primary antibody complex may also be raised in a conventional fashion, e.g. by mouse immunization followed by fusion with myeloma cells, etc.

In the assay method of the invention, the aqueous reagents are preferably contacted with the sample sequentially with the analyte determination taking place after addition of the final reagent. However, unlike earlier assays, if the time of determination is carefully controlled, determination may take place before complex generation is complete: in this way the time required for performing the assay may be reduced, e.g. to less than 15 minutes, advantageously less than 10 minutes and especially advantageously less than 5 minutes.

The sample investigated using the method of the

invention may be any homocysteine containing sample. Generally it will be a body fluid or a liquid derived from a body fluid, e.g. blood, urine, cerebrospinal fluid, serum or plasma. Serum is preferred and plasma is particularly preferred. Where the sample is one in which homocysteine is protein bound (e.g. blood, serum, plasma, etc.) it is preferred that one of the reagents, preferably the first reagent, added to the sample, should contain a reducing agent so as to liberate the free reduced homocysteine. Dithiothreitol (DTT) and tris(2-carboxyethyl)phosphine (TCEP) are preferred as reductants. TCEP is especially preferred since it is a strong reductant which is stable at pH values of about 7. Other pH 7 stable reductants could however be used if desired.

The first reagent also preferably contains the co-substrate for the homocysteine converting enzyme, if such a co-substrate is required. Thus for example where the homocysteine converting enzyme is SAH-hydrolase it is desirable to include adenosine or an adenosine analog in the reagents.

In general one or more of the reagents will preferably contain an agent which promotes precipitation of the polyhapten:primary antibody complex. Suitable such materials include polysaccharides, polyhydroxyl compounds, carbohydrates, alginates, chitosan, heparin, heparinoids, poloxamers and poloxamines. Especially preferred are polyalkylene oxides such as polyethylene glycol (PEG). PEG of molecular weight 1 to 10 kD, particularly 2 to 8 kD and more particularly about 6 kD, functions particularly well in this regard.

Besides agents such as PEG, complex precipitation may be enhanced by reducing pH to acid levels. This however is less preferred.

Less preferably, immunocomplex precipitation may be enhanced using organic solvents or salts, e.g. ammonium acetate, metal cations, 2-ethyl-6,9-diaminoacridine

lactate, protamine, polyacrylates, etc.

Since agents which promote complex precipitation may also promote precipitation of other proteins present in the sample, where such agents are used they are preferably present in two or all three of the reagents.

Depending on whether two or three reagents are used according to the invention, the different components may be distributed differently in the reagents. For a two reagent system, the first reagent desirably contains an immunocomplex precipitation promoter (e.g. PEG), a co-substrate (e.g. adenosine) if such is necessary, a reductant (e.g. TCEP), and a polyhapten (e.g. poly-SAH), while the second reagent desirably contains the primary antibody (e.g. anti-SAH), the homocysteine converting enzyme (e.g. SAH-hydrolase), a complex precipitation promoter (e.g. PEG) and, if desired, the secondary antibody. Where three reagents are used, two or three preferably contain an immunocomplex precipitation promoter (e.g. PEG); while the primary antibody and the homocysteine converting enzyme are preferably not formulated together with the polyhapten or, where present, the co-substrate.

If desired the reagents used may include a carrier protein, e.g. casein or albumin, both as a stabilizer and to compensate for variations in the background signal from proteins in the sample (e.g. plasma proteins) which may occur at varying levels in different samples - by adding carrier protein the percentage variation between samples in the proteins responsible for background signal is reduced.

In the assay method of the invention, an analyte competes with the polyhapten for binding to the primary antibody. The analyte may be a homocysteine co-substrate or a homocysteine conversion product or (where a second enzyme is used) a conversion product of any of these. The polyhapten preferably is a water-soluble compound or compound mixture which has at least two,



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preferably at least 5, more preferably at least 10 and especially preferably at least 50 hapten moieties which are the same as or similar to at least part of the analyte such that the primary antibody is capable of binding both to the analyte and the hapten moieties of the polyhapten. More generally, the polyhapten preferably has at least one hapten moiety per 100 kD of its molecular weight and the polyhapten preferably has a molecular weight of at least 500 kD, more preferably at least 1 MD, e.g. up to 3 MD. Conveniently, it has a polymeric backbone structure onto which the haptens are bound, e.g. a polypeptide structure (for example a protein such as a thyroglobulin or a polypeptide such as polyaspartic acid), a polysaccharide (such as a dextran derivative, alginic acid and mannan), or a synthetic polymer with pendant functional groups such as hydroxyl, carboxyl and/or amino groups. Porcine thyroglobulin, which has a molecular weight of 660 kD, is especially preferred since its size is appropriate and since it can be dimerized to produce a larger polyhapten. In general proteins and polypeptides will be preferred as polyhapten backbone materials since their isoelectric points, and hence the precipitation characteristics of the polyhapten:primary antibody complex, can readily be modified. The hapten moieties may be coupled to the polyhapten backbone using conventional chemistry, e.g. using peptide coupling between amine and carboxyl groups of the hapten and the polyhapten-backbone compound.

Thus, by way of example, SAH can be coupled to porcine thyroglobulin using EDC/EDAC (1-ethyl-3-(dimethylaminopropyl)-carbodiimide) or another carboxyl activator, optionally after treating the protein with succinic acid. A one-pot reaction is possible, but it is preferred to succinylate the protein in a separate step before conjugation to SAH. Succinic acid couples to free amino groups on the protein, provides a pendant carboxyl for SAH attachment and also lowers the

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isoelectric point of the protein. The carboxyl activator permits coupling of SAH carboxyls with protein amino groups as well as of SAH amino groups with native and succinic acid-deriving carboxyls on the proteins. In such a reaction, N-hydroxy succinimide may also be used to promote SAH:protein coupling.

As mentioned above, the polyhapten is preferably water-soluble. However it is also possible to use colloidal particles, for example nano-particles, e.g. particles having sizes in the 1 to 1000 nm, especially 50 to 800 nm range, as a matrix onto which the haptens are bound. The particle size should be such that the particles form a stable aqueous dispersion from which they do not settle out. The particles may be of a synthetic polymer with suitable functional groups for hapten attachment.

The homocysteine converting enzyme used in the assay method may be any enzyme capable of directly or indirectly using or generating an antigenic analyte. Typical enzymes include SAH-hydrolase, homocysteinase (homocysteine desulphurase), methionine synthase, cystathionine  $\beta$ -synthase, and betaine-homocysteine methyl transferase. Where the enzyme used is SAH-hydrolase, the analyte will generally be adenosine or SAH or an enzymic conversion product of one of these; however SAH is the preferred analyte. Where the enzyme used is homocysteine desulphurase, the analyte will generally be an enzyme conversion product of  $\alpha$ -ketobutyrate.

In the assay method of the invention, a secondary antibody which binds to the polyhapten:primary antibody complex may be used if desired. In this way precipitation of the polyhapten:primary antibody complex is enhanced and the sensitivity of the assay may be improved. The secondary antibody may for example be an anti-mouse IgG polyclonal antibody where the primary antibody is a mouse antibody. The secondary antibody

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may be polyclonal or monoclonal. The secondary antibody can be of any origin, e.g. rabbit, chicken, etc., as long as its cross-reactivity with components in the sample under investigation is not such as to cause any change in light transmission or scattering. Thus cross-reactivity with human plasma proteins and antibodies is desirably absent.

The secondary antibody need not be a traditional antibody: any specific binding material which enhances precipitation of the polyhapten:primary antibody complex may be used, e.g. antibody fragments, oligopeptides, oligonucleotides, single chain antibodies, etc., optionally with two or more coupled together.

The use of secondary antibodies however potentially increases the compatibility problems, ie. the problems faced in producing an assay method requiring only two or three reagents. For a three reagent system, it is possible to have primary and secondary antibodies and polyhapten in separate reagents. However, it is possible to formulate the primary and secondary antibodies together if a chaotropic salt is also included in the reagent. Such chaotropic salts include for example salts containing  $\text{SCN}^-$ ,  $\text{CCl}_3\text{COO}^-$ ,  $\text{CF}_3\text{COO}^-$ ,  $\text{Cs}^+$ ,  $\text{Li}^+$ , or  $\text{Mg}^{2+}$  ions and guanidine chloride.

The twin antibody reagent will preferably also contain a reagent which promotes precipitation of the immunocomplex, e.g. a polyalkylene oxide such as PEG or other such compounds as discussed earlier. Thus the antibodies, PEG and chaotropic salt will be at a higher concentration than in the reaction medium when they are mixed with sample and polyhapten. By combining a small volume of this antibody containing reagent with a larger volume of another solution, e.g. another reagent mixed with the sample, dilution of the chaotropic salt will permit immunoprecipitation to begin. This novel strategy can be used in other assays to obtain signal enhancement and forms a further aspect of the invention.

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Viewed from this aspect, the invention provides an aqueous assay reagent containing a chaotropic salt, an immuno-precipitation enhancer (e.g. PEG), a primary antibody and a secondary antibody.

The invention will now be illustrated further by reference to the following non-limiting Examples and to the accompanying drawings, in which:

Figure 1 shows a plot of absorption against time for calibration samples under the assay system of Example 8; Figure 2 shows a plot of absorption against time for calibration samples under the assay system of Example 9; Figure 3 shows dose response curves for the assays using the polyhaptenes of Examples 4 and 5; Figure 4 shows a comparison of immunoprecipitation signal obtained using BSA-SAH, IgY-SAH and PTG-SAH conjugates under the assay conditions of Example 8; Figure 5 shows a plot of absorption against time for calibration samples under the assay system of Example 10; and Figure 6 shows the dose response curve for the assay of Example 10.

#### EXAMPLE 1

##### Primary Antibody Production

Balb/c mice were immunised with SAH coupled to bovine serum albumin. After the fourth injection, splenocytes from the mice were fused with myeloma cells as described by Gaffre et al. "Preparation of Monoclonal Antibodies: Strategies and Procedures", Meth. Enzymology pages 31-46 (1987). The hybrid cell supernatants were screened for binding to SAH and BSA and those binding to SAH but not BSA were selected for cloning. After stabilising the clone, the cells were grown *in vitro* in hollow fibre systems and the antibodies were purified from the

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supernatant using Protein A-sepharose column chromatography (see Ey et al. Immunochem. 15: 429-436 (1978)).

## EXAMPLE 2

### Secondary Antibody Production

Commercially available polyclonal anti-mouse IgG antibodies may be used. These are produced by injecting mouse IgG into another species (e.g. chicken, goat, rabbit, etc), harvesting antisera and purifying the harvested antisera by immunoaffinity chromatography using antigens coupled to agarose gels.

In the following Examples the secondary antibody is LAJD-112 from La Jolla Diagnostics Inc, California.

(The secondary antibody may alternatively be a monoclonal antibody against the polyhapten:primary antibody complex. In this event it may be produced analogously to the antibody of Example 1 using in place of the SAH-bovine serum albumin conjugate the polyhapten:primary antibody complex or a fragment thereof including the binding site between the polyhapten and the primary antibody).

## EXAMPLE 3

### Polyhapten production

(A) 150  $\mu$ L succinic anhydride (7.5 mg/mL in N,N-dimethylformamide) was added dropwise to 5 mL of porcine thyroglobulin (PTG) (5 mg/mL in 10 mM phosphate buffer, pH 7.4). The mixture was allowed to react for 4 hours at 20-25°C with constant stirring.

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(B) The product of step (A) was dialysed against 2L of 2-morpholinoethanesulphonic acid (MES) buffer, pH 5.5.

(C) 10 mL of the modified PTG from step (B) (approx. 5 mg/mL in the MES buffer) was mixed with 6.8 mL SAH solution (17 mg in 6.8 mL MES buffer) and 0.7 mL sulpho-N-hydroxysuccinimide solution (5 mg in 1 mL MES buffer).

(D) With constant stirring, 7.6 mg (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.HCl (EDC) was added and the mixture was allowed to react at 20-25°C for 3-5 hours.

(E) The product of step (D) was dialysed against 5L of 10 mM phosphate buffer, pH 7.4. The PTG-polySAH product is used as a polyhaptten.

Bovine serum albumin-SAH polyhaptten (BSA-SA-SAH) is produced by succinylation followed by carbodiimide coupling using water soluble or insoluble carbodiimides, e.g. EDC/EDAC, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide-p-toluene sulphonate, or dicyclohexylcarbodiimide, IgG-SAH and IgY (chicken immunoglobulin)-SAH polyhaptens are produced analogously. Dextran-SAH polyhaptten is prepared by reaction of amino-dextran with succinic anhydride followed by SAH coupling using a carbodiimide. Alginic acid-SAH is prepared by carbodiimide coupling analogously to steps (D) and (E) in Example 3.

### EXAMPLE 3

#### Polyhaptten production

(A) 120 µl succinic anhydride (7.5 mg/mL in N,N-dimethylformamide (DMF)) is added dropwise to 1 ml of poly-L-glutamic acid (10 mg/mL dissolved in 10 mM

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phosphate buffer, pH 7.4). The mixture is allowed to react for 4 hours at 20-25°C with constant stirring.

(B) The resulting modified (N-terminal blocked) polyglutamic acid is dialysed against 1L of MES buffer, pH 5.5.

(C) 1.6 ml of the dialysed product (approx. 7 mg/ml in MES buffer) is mixed with 0.8 ml SAH solution (1.9 mg/mL in 100 mM MES Buffer, pH 5.5) and 0.8 ml sulfo-N-hydroxysuccinimide solution (0.5 mg in 1 mL 100 mM MES buffer, pH 5.5).

(D) With constant stirring, 22 mg EDC is added. The mixture is allowed to react for 3 to 5 hours at 20-25°C.

(E) The product (s-poly-Glu-SAH) is dialysed against 3L of 10 mM phosphate buffer, pH 7.4.

#### EXAMPLE 5

##### Polyhapten production

(A) 150 µl succinic anhydride (7.5 mg/ml in DMF) is added dropwise to 2 ml of casein (5 mg/ml in 10 mM phosphate buffer, pH 7.4). The mixture is allowed to react for 4 hours at 20-25°C with constant stirring.

(B) The resulting modified casein is dialysed against 1L of MES buffer, pH 5.5.

(C) 1 ml of the modified casein (approx. 4 mg/ml in 100 mM MES buffer, pH 5.5) is mixed with 0.5 ml SAH solution (2.5 mg/mL in 100 mM MES Buffer, pH 5.5) and 0.07 ml sulfo-N-hydroxysuccinimide solution (5 mg in 1 mL 100 mM MES buffer, pH 5.5).

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(D) With constant stirring, 1.2 mg EDC is added. The mixture is allowed to react for 3 to 5 hours at 20-25°C.

(E) The product (s-casein-SAH) is dialysed against 3L of 10 mM phosphate buffer, pH 7.4.

#### EXAMPLE 6

##### Polyhapten preparation

(A) 150  $\mu$ l succinic anhydride (100  $\mu$ mol/ml in DMF) is added dropwise to 2 ml of IgY (chicken antibody, 46 mg/ml in 10 mM PBS-buffer, pH 7.4). The mixture is allowed to react for 4 hours at 20-25 °C with constant stirring.

(B) The resulting modified IgY is dialysed against 10mM MES-buffer, pH 5.5.

(C) 1 ml of the modified IgY (approx. 4 mg/ml in 100 mM MES buffer, pH 5.5) is mixed with 0.5 ml SAH solution (2.5 mg/mL in 100 mM MES Buffer, pH 5.5) and 0.07 ml sulpho-N-hydroxysuccinimide solution (5 mg in 1 mL 100 mM MES buffer, pH 5.5).

(D) With constant stirring, 1.2 mg EDC is added. The mixture is allowed to react for 3 to 5 hours at 20-25°C.

(E) The product (sIgY-SAH) is dialysed against 3 L of 10mM PBS-buffer.

#### EXAMPLE 7

##### Polyhapten preparation

(A) 1 mL of 0.1M NaIO<sub>4</sub> is added dropwise to 1 ml mannan (5 mg/ml in water). The mixture is allowed to react for



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30 minutes at 20-25°C with constant stirring.

(B) The modified mannan is dialysed against 1L of 10 mM carbonate-buffer, pH 9.5.

(C) 1 mL of the modified mannan (approx. 4 mg/ml in 10 mM carbonate buffer, pH 9.5) is mixed with 1 ml SAH solution (1.9 mg/ml in 10 mM carbonate buffer, pH 9.5). The temperature is maintained at 2-10°C and the reaction is allowed to proceed for 10 hours.

(D) 20 µl 0.1M aqueous NaBH<sub>4</sub> is added and the mixture is incubated at 20-25°C for 3 hours.

(E) The mannan-SAH conjugate is dialysed against 1.5L of 10 mM phosphate buffer, pH 7.4.

Figure 4 of the accompanying drawings shows a comparison of the immunoprecipitation signal detected using sBSA-, sIgY-, and sPTG-SAH conjugates as the polyhapten. As can be seen, the precipitation properties of the different conjugates do vary.

The immunoprecipitation ability of the polyhaptens of the Examples is PTG-poly-SAH > sIgY-SAH > mannan-SAH > BSA-SA-SAH = s-poly-Glu-SA > s-Casein-SAH > alginic acid-SAH.

#### EXAMPLE 8

##### Two Reagent Assay

##### Reagent 1

10 mM phosphate buffer\*, 0.15M NaCl, pH 7.4

30% w/v PEG 6000

10% Triton X-100 (aqueous)

4800 µL —

1200 µL

350 µL

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2 mM Adenosine (in phosphate buffer*)	15 $\mu$ L
50 mM TCEP (aq)	140 $\mu$ L
PTG-SAH polyhapten (2mg/mL)	400 $\mu$ L

Reagent 2

10 mM phosphate buffer*	730 $\mu$ L
30% w/v PEG 6000	270 $\mu$ L
Primary antibody (3 mg/mL)	400 $\mu$ L
SAH hydrolase ( $\approx$ 350 U/mL)	600 $\mu$ L

Assay Protocol

10  $\mu$ L sample (e.g. human plasma) is mixed with 180  $\mu$ L of Reagent 1 and incubated for 0.25 to 5 minutes at 37°C. 50  $\mu$ L of Reagent 2 is added, the mixture is incubated for 3 to 10 minutes at 37°C and the precipitation signal is determined by absorption measurements at 340-450 nm, preferably 340 nm.

The assay is calibrated with L-homocyst(e)ine standards.

The total assay time can be as little as 3 minutes. Figure 1 of the accompanying drawings shows a plot of absorption vs time for calibration samples. The jump at measuring point 12 marks the addition of Reagent 2. The assay time may be shortened by careful timing of Reagent addition and absorption measurement and using the difference between measurement at two times after addition of Reagent 2 (e.g. measuring points 15 and 21 in Figure 1). This may be done even though the signal strength may not have reached its maximum at the later point.

EXAMPLE 9Two Reagent Assay

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Reagent 1

As Example 8

Reagent 2

10 mM phosphate buffer*	625 $\mu$ L
30% w/v PEG 600	270 $\mu$ L
Primary antibody (3 mg/mL)	400 $\mu$ L
SAH hydrolase ( $\approx$ 350 U/mL)	600 $\mu$ L
5M NaSCN (aq.)	70 $\mu$ L
Secondary antibody (rabbit-anti-mouse) (2 mg/mL)	35 $\mu$ L

Assay Protocol

As for Example 8

Fig. 2 of the accompanying drawings shows a plot of absorption vs. time for a series of calibration samples.

The dose response curve for the assays using the polyhaptens of Examples 4 and 5 are shown in Figure 3 of the accompanying drawings. These are obtained by plotting the absorption difference (dA 340 nm) between the measurements at measuring points 15 and 21 (ie. 7.5 and 10.5 minutes).

EXAMPLE 10Two Reagent AssayReagent 1

10 mM Bis-Tris, pH 6.4	190 $\mu$ L
Triton X-100 (aqueous)	50 $\mu$ L
0.5 mM Adenosine (aqueous)	128 $\mu$ L

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5 mM TCEP in Bis-Tris pH 6.4	60 $\mu$ L
water	233 $\mu$ L
1M NaSCN (aqueous)	50 $\mu$ L
1.5 M NaCl (aqueous)	79 $\mu$ L
PTG-SAH polyhapten ( $\approx$ 2mg/mL)	210 $\mu$ L

Reagent 2

10% PEG (aqueous)	900 $\mu$ L
100 mM phosphate-buffer, pH 7.4	210 $\mu$ L
water	140 $\mu$ L
1.5 M NaCl	220 $\mu$ L
SAH hydrolase ( $\approx$ 350 U/mL)	230 $\mu$ L
Primary antibody (3 mg/mL)	300 $\mu$ L

Assay Protocol

10  $\mu$ L sample (e.g. human plasma) is mixed with 50  $\mu$ L of Reagent 1 and incubated for 5 minutes at 37°C. 200  $\mu$ L of Reagent 2 is added, the mixture is incubated for 5 minutes at 37°C and the precipitation signal is determined by absorption measurements at 340-450 nm, preferably 340 nm.

The assay is calibrated with L-homocyst(e)ine standards.

Figure 5 of the accompanying drawings shows a plot of absorption vs time for calibration samples. Dose response curves can be derived from the signal difference between points over the final 5 minutes of the reaction. The dose response curve shown in Figure 6 is derived by plotting the absorption difference between points 13 and 18 as a function of calibrator concentration.